Quantibody® Human Periodontal Disease Array 1

Quantitative measurement of 20 human periodontal disease associated cytokines

Catalog #: QAH-PDD-1

User Manual
Last revised May, 2015

Caution:
Extraordinarily useful information enclosed

ISO 13485 Certified

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Please read the entire manual carefully before starting your experiment

I. Overview

Cytokines Detected (20)	CRP (C-Reactive Protein), IFN-gamma, IL-1 alpha (IL-1 F1), IL-1 beta (IL-1 F2), IL-10, IL-12 p70, IL-17A, IL-2, IL-4, IL-6, IL-8 (CXCL8), MIP-1 alpha (CCL3), MMP-13, MMP-9, Osteoactivin (GPNMB), Osteopontin (SPP1), Osteoprotegerin (TNFRSF11B), RANK (TNFRSF11A), TGF beta 1, TNF alpha *See Section IX for Array Map*
Format	One standard glass slide is spotted with 16 wells of identical cytokine antibody arrays. Each antibody is arrayed in quadruplicate.
Detection Method	Fluorescence. Go to www.RayBiotech.com/Scanners for a list of compatible laser scanners.
Sample Volume	50 - 100 µl per array
Reproducibility	CV <20%
Assay Duration	6 hours

II. Introduction

Periodontal disease is a gum disease. The symptom ranges from simple gum inflammation (gingivitis) to periodontitis which results in major damage to the soft tissue and bone that support the teeth. In the worst cases, teeth are lost. Because of the irreversible nature of periodontitis, early diagnosis and treatment is critical. Clinical measurements include probing pocket depth, bleeding on probing, clinical attachment loss, plaque index, and radiographs etc. While such methods are useful for the staging of periodontal disease, they are only indicators of previous disease status rather than the present disease activity. There is a need for the development of new diagnostic tests that can reflect the active disease state, which will be useful for disease diagnosis, prognosis, and monitoring the effectiveness of periodontal therapy. Gingival crevicular fluid (GCF) is a tiny amount bodily fluid transuded from periodontal tissues into the gingival crevice and periodontal pocket. The constituents of GCF originate from serum, gingival tissues, and from both bacterial and host response cells, which reflect the biology and physiology of the local tissues. Meanwhile, GCF could be easily collected by noninvasive means such as paper strips, absorbent points and micropipettes. As a result, proteins in GCF have been the ideal and hot targets pursued for candidate disease

specific biomarker research for the last several decades.

Most analyzed periodontal disease related proteins in GCF are inflammatory cytokines (eg. IL-1b, IL-6, IL-8, IL-10, IL-12, IFNg, TNFa, and CRP); matrix metalloproteinases (eg. MMP-8, MMP-9, and MMP-13) and their inhibitors (TIMPs); bone metabolism related cytokines (eg. OPG, OPN, RANK, and RANKL); and enzymes (eg. alkaline phosphatase and aspartate aminotransferase). Because of the limited availability of sample volumes, most of the previous research only worked on one or several targets. The traditional method for cytokine detection and quantification is through the use of an enzyme-linked immunosorbent array (ELISA). While the traditional method works well for a single protein, the overall procedure is time consuming and requires a lot of sample. Take the advantage of advancement in microarray technology over the last decade; Raybiotech, has pioneered the development of cytokine antibody arrays, which has now been widely applied in the research community with hundreds of peer reviewed publications such as in Cell and Nature.The traditional method for cytokine detection and quantification is through the use of an enzyme-linked immunosorbent assay (ELISA). In this method, target protein is immobilized to a solid support. The immobilized protein is then complexed with an antibody that is linked to an enzyme. Detection of the enzyme complex can then be visualized through the use of a substrate that produces a detectable signal. While this traditional method works well for a single protein, the overall procedure is time consuming and requires a relatively high volume of sample. Thus, conservation of precious small sample quantities becomes a challenging task. Innovations in microarray technology over the last decade have addressed this problem. A long-standing leader in the field, Raybiotech, has pioneered the development of cytokine antibody arrays, which have now been widely applied in the research community with hundreds of peer reviewed publications, including top-tier journals such as *Cell* and *Nature*.

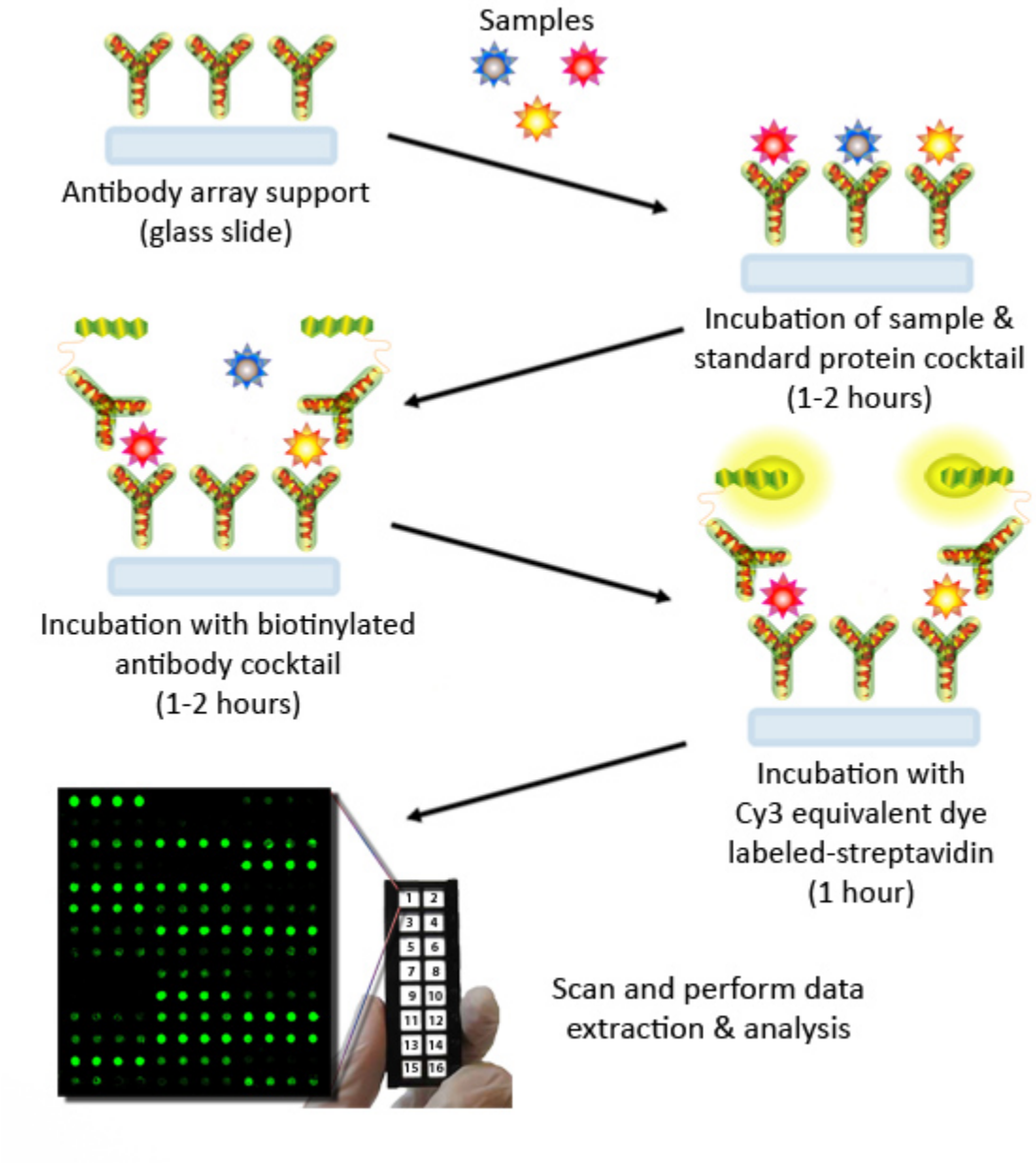
The Quantibody® array, our multiplexed sandwich ELISA-based quantitative array platform, enables researchers to accurately determine the concentration of multiple cytokines simultaneously. It combines the advantages of the high detection sensitivity & specificity of ELISA and the high throughput of arrays. Like a traditional sandwich-based ELISA, it uses a pair of cytokine specific antibodies for detection. A capture antibody is first bound to the glass surface. After incubation with the sample, the target cytokine is trapped on the solid surface. A second biotin-labeled detection antibody is then added, which can recognize a different epitope of the target cytokine. The cytokine-antibody-biotin complex can then be visualized through the addition of the streptavidin-conjugated Cy3 equivalent dye, using a laser scanner. Unlike the traditional ELISA, Quantibody products use an array format. By arraying multiple cytokine specific capture antibodies onto a glass support, quantitative, multiplex detection of cytokines in one experiment is made possible.

In detail, one standard glass slide is divided into 16 wells of identical cytokine antibody arrays. Each antibody, together with the positive controls is arrayed in quadruplicate. The slide comes with a 16-well removable gasket which allows for the process of 16 samples on one slide. Four slides can be nested into a tray, which matches a standard microplate footprint and allows for automated robotic high throughput process of 64 arrays

simultaneously. For cytokine quantification, the array specific cytokine standards, whose concentration has been predetermined, are provided to generate a standard curve for each cytokine. In a real experiment, standard cytokines and samples will be assayed in each array simultaneously through a sandwich ELISA procedure. By comparing signals from unknown samples to the standard curve, the cytokine concentration in the samples will be determined.

Quantibody® array kits have been confirmed to have similar detection sensitivity as traditional ELISA. Our current high density Quantibody kits allow scientists to quantitatively determine the concentration of 660 human, 200 mouse, and 67 rat cytokines in a single experiment. This is not only one of the most efficient products on the market for cytokine quantification, but makes it more affordable for quantification of large number of proteins. Simultaneous detection of multiple cytokines undoubtedly provides a powerful tool for drug and biomarker discovery.

III. How It Works



IV. Materials Provided

	Catalog #	Component Name	1 Slide Box	2 Slide Box*
1	QAH-PDD-1S	Human Periodontal Disease Array 1 Glass Slide	1	2
2	QA-SDB	Quantibody® Sample Diluent	15 ml	
3	AA-WB1-30ML	20X Wash Buffer I	2 x 30 ml	3 x 30 ml
4	AA-WB2-30ML	20X Wash Buffer II	30 ml	
5	QAH-PDD-1-STD	Human Periodontal Disease Array 1 Lyophilized Standard Mix**	1 Vial	
6	QAH-PDD-1B	Human Periodontal Disease Array 1 Biotinylated Antibody Cocktail	1-25 µl	2 x 1-25 µl
7	QA-CY3E	Cy3 equivalent dye-conjugated Streptavidin	5 µl	2 x 5 µl
8	QA-SWD	Slide Washer/Dryer	1 x 30 ml Tube	
9	QA-ADH	Adhesive Film	1	2

* 4 slide kits are comprised of 2 separate 2 slide kits.
** See Section X for detailed cytokine concentrations after reconstitution.

V. Storage

Upon receipt, all components should be stored at -20°C. The kit will retain activity for up to 6 months. Once thawed, the glass slide, standard mix, antibody cocktail and dye-conjugated Streptavidin should be kept at -20°C. All other components may be stored at 4°C. The entire kit should be used within 6 months of purchase.

VI. Additional Materials Required

- Benchtop rocker or orbital rocker
- Laser scanner for fluorescence detection
- Aluminum foil
- Distilled water
- 1.5 ml Polypropylene microcentrifuge tubes

VII. General Considerations

A. Preparation of Samples

- Use serum-free conditioned media if possible.
- If serum-containing conditioned media is required, it is highly recommended that complete medium be used as a control since many types of sera contains cytokines.
- We recommend the following parameters for your samples: 50 to 100 µl of original or diluted serum, plasma, cell culture media, or other body fluid, or 50-500 µg/ml of protein for cell and tissue lysates.

  If you experience high background or if the fluorescent signal intensities exceed the detection range, further dilution of your sample is recommended.

B. Handling Glass Slides

- Do not touch the surface of the slides, as the microarray slides are very sensitive. Hold the slides by the edges only.
- Handle all buffers and slides with powder free gloves.
- Handle glass slide/s in clean environment.
- The Quantibody slides do not have bar codes. To help distinguish one slide from another, transcribe the slide serial number from the slide bag to the back of the slide with a fine point permanent marker. Please write the number on the very bottom edge of the slide, taking care to avoid writing on the array well areas.

C. Incubation

- Completely cover array area with sample or buffer during incubation.
- Avoid foaming during incubation steps.
- Perform all incubation and wash steps under gentle rocking or rotation.
- Cover the incubation chamber with adhesive film during incubation, particularly when incubation is more than 2 hours or <70 µl of sample or reagent is used.
- Several incubation steps such as step 6 (blocking), step 7 (sample incubation), step 10 (detection antibody incubation), or step 13 (Cy3 equivalent dye-streptavidin incubation) may be done overnight at 4°C. Please make sure to cover the incubation chamber tightly to prevent evaporation.

VIII. Protocol

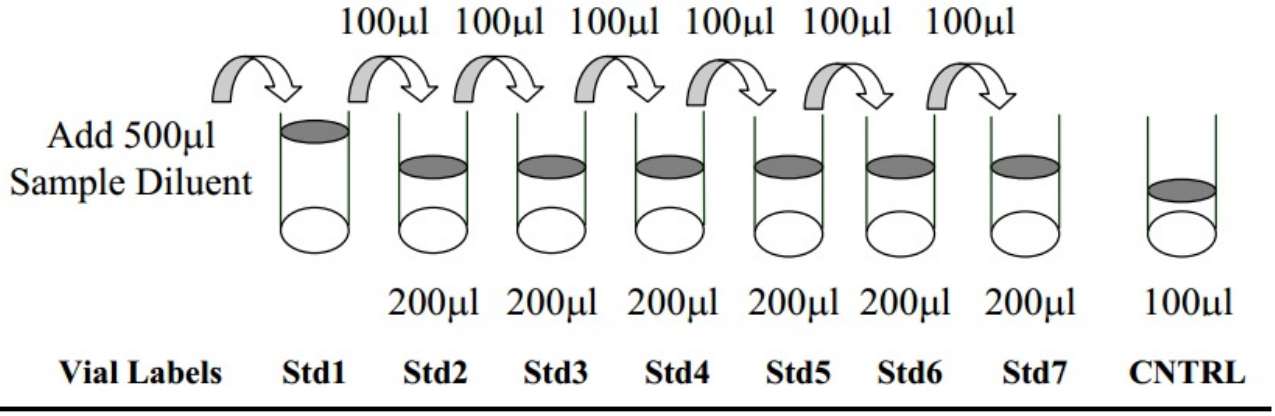
A. Completely Air Dry The Glass Slide

1. Take out the glass slide from the box, and let it equilibrate to room temperature inside the sealed plastic bag for 20-30 minutes. Remove slide from the plastic bag, peel off the cover film, and let it air dry for another 1-2 hours.

Incomplete drying of slides before use may cause the formation of "comet tails," thin directional smearing of antibody spots.

B. Prepare Cytokine Standard Dilutions

There is only one vial of standard provided in the two-slide kit, which is enough for making two standard curves. Reconstitute the lyophilized standard within one hour of usage. If you must use the standard for two different days, store only the Std1 dilution at -80°C.


100µl 100µl 100µl 100µl 100µl 100µl
Add 500µl
Sample Diluent
200µl 200µl 200µl 200µl 200µl 200µl 100µl
Vial Labels Std1 Std2 Std3 Std4 Std5 Std6 Std7 CNTRL

2. Reconstitute the Cytokine Standard Mix (lyophilized) by adding 500 µl Sample Diluent to the tube. For best recovery, always quick-spin vial prior to opening. Dissolve the powder thoroughly by a gentle mix. Labeled the tube as Std1.

3. Label 6 clean microcentrifuge tubes as Std2 to Std7. Add 200 µl Sample Diluent to each of the tubes.

4. Pipette 100 µl Std1 into tube Std2 and mix gently. Perform 5 more serial dilutions by adding 100 µl Std2 to tube Std3 and so on.

5. Add 100 µl Sample Diluent to another tube labeled as CNTRL. Do not add standard cytokines or samples to the CNTRL tube, which will be used as negative control. For best results, include a set of standards in each slide.

Since the starting concentration of each cytokine is different, the serial concentrations from Std1 to Std7 for each cytokine are varied which can be found in Section X.

C. Blocking & Incubation

6. Add 100 µl Sample Diluent into each well and incubate at room temperature for 30 minutes to block slides.

7. Decant buffer from each well. Add 100 µl standard cytokines or samples to each well. Incubate arrays at room temperature for 1-2 hour.

Longer incubation time is preferable for higher signals. This step may be done overnight at 4°C.

We recommend using 50 to 100 µl of original or diluted serum, plasma, conditioned media, or other body fluid, or 50-500 µg/ml of protein for cell and tissue lysates. Cover the incubation chamber with adhesive film during incubation, especially if less than 70 ul of sample or reagent is used.

8. Wash:

- Decant the samples from each well, and wash 5 times (5 min each) with 150 µl of 1X Wash Buffer I at room temperature with gentle rocking. Completely remove wash buffer in each wash step. Dilute 20x Wash Buffer I with H2O.

- *(Optional for Cell and Tissue Lysates)* Put the glass slide with frame into a box with 1X Wash Buffer I (cover the whole glass slide and frame with Wash Buffer I), and wash at room temperature with gentle rocking for 20 min.

- Decant the 1x Wash Buffer I from each well, wash 2 times (5 min each) with 150 µl of 1X Wash Buffer II at room temperature with gentle rocking. Completely remove wash buffer in each wash step. Dilute 20X Wash Buffer II with H2O.

Incomplete removal of the wash buffer in each wash step may cause "dark spots," the background signals higher than the spots.

D. Incubation with Biotinylated Antibody Cocktail & Wash

9. Reconstitute the detection antibody by adding 1.4 ml of Sample Diluent to the tube. Spin briefly.

10. Add 80 µl of the detection antibody cocktail to each well. Incubate at room temperature for 1-2 hour.

Longer incubation time is preferable for higher signals and backgrounds

11. Decant the samples from each well, and wash 5 times (5 mins each) with 150 µl of 1X Wash Buffer I and then 2 times with 150 µl of 1x Wash Buffer II at room temperature with gentle rocking. Completely remove wash buffer in each wash step.

E. Incubation with Cy3 Equivalent Dye-Streptavidin & Wash

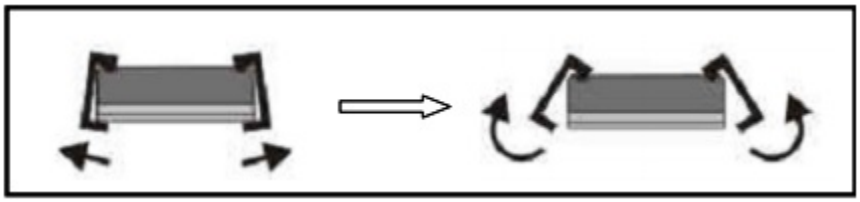
12. After briefly spinning down, add 1.4 ml of Sample Diluent to Cy3 equivalent dye-conjugated streptavidin tube. Mix gently.

13. Add 80 µl of Cy3 equivalent dye-conjugated streptavidin to each well. Cover the device with aluminum foil to avoid exposure to light or incubate in dark room. Incubate at room temperature for 1 hour.

14. Decant the samples from each well, and wash 5 times (5 mins each) with 150 µl of 1X Wash Buffer I at room temperature with gentle rocking. Completely remove wash buffer in each wash step.

F. Fluorescence Detection

15. Disassemble the device by pushing clips outward from the slide side. Carefully remove the slide from the gasket.



Be careful not to touch the surface of the array side.

16. Place the slide in the Slide Washer/Dryer (a 4-slide holder/centrifuge tube), add enough 1x Wash Buffer I (about 30 ml) to cover the whole slide, and then gently shake at room temperature for 15 minutes. Decant Wash Buffer I. Wash with 1x Wash Buffer II (about 30 ml) and gently shake at room temperature for 5 minutes.

17. Remove water droplets completely by gently applying suction with a pipette to remove water droplets. Do not touch the array, only the sides.

    You may also dry the glass slide by a compressed N_2 stream.

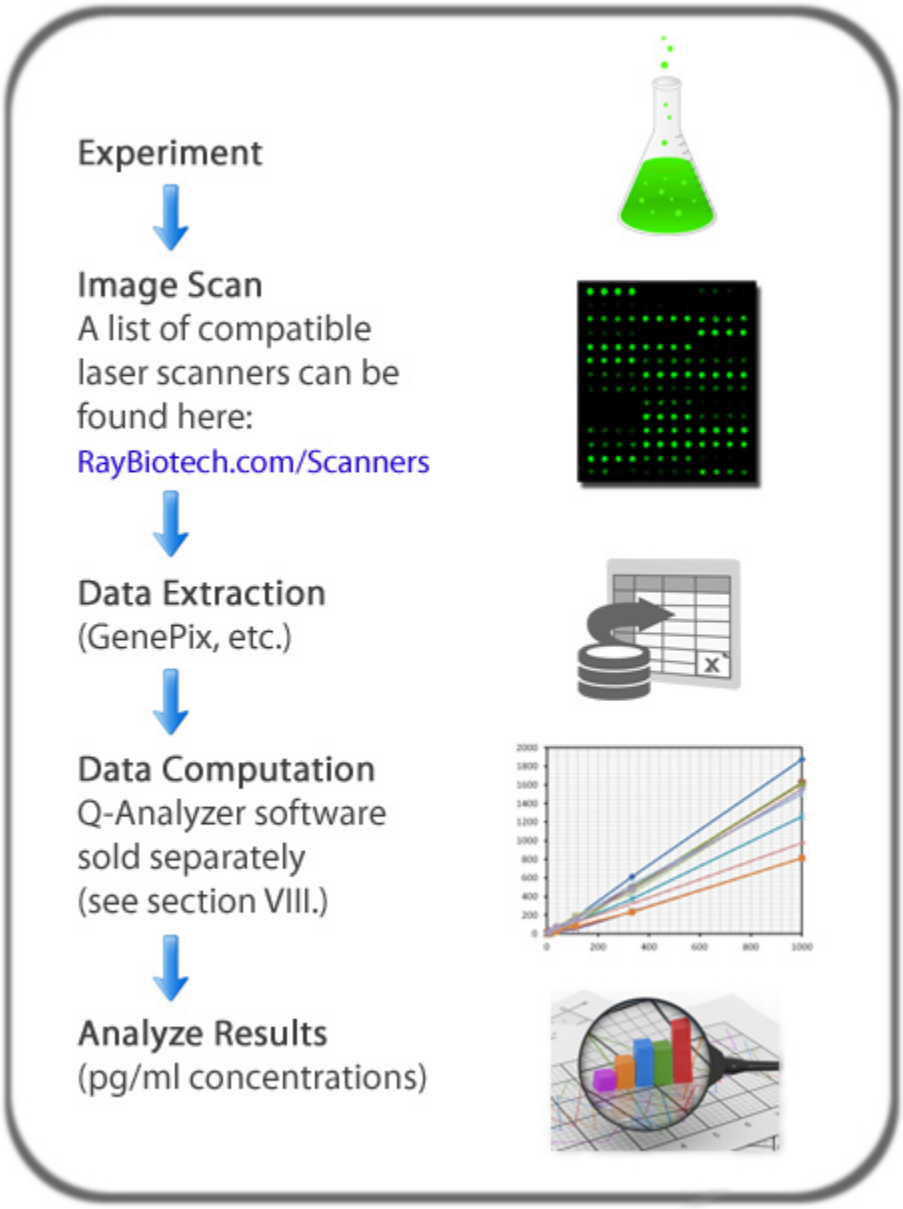
18. Imaging: The signals can be visualized through use of a laser scanner equipped with a Cy3 wavelength (green channel) such as Axon GenePix. Make sure that the signal from the well containing the highest standard concentration (Std1) receives the highest possible reading, yet remains unsaturated.

In case the signal intensity for different cytokine varies greatly in the same array, we recommend using multiple scans, with a higher PMT for low signal cytokines, and a low PMT for high signal cytokines.

G. Data Analysis

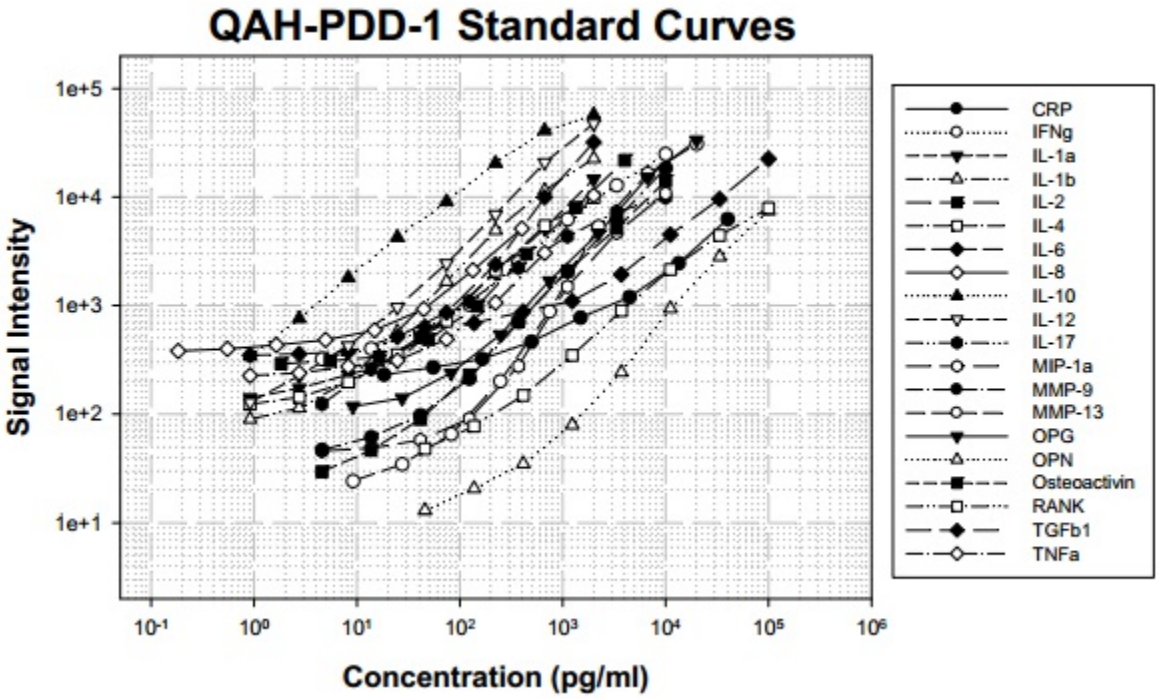
19. Data extraction can be done using the GAL file that is specific for this array along with the microarray analysis software (GenePix, ScanArray Express, ArrayVision, MicroVigene, etc.). GAL files can be found here: www.RayBiotech.com/Gal-Files.html.

    Need help analyzing all that data? Copy and paste your data into the Q-Analyzer Tool specific for this array, catalog number: **QAH-PDD-1-SW**. More information can be found in Section XII.



IX. Array Map & Standard Curves

Each antibody is printed in quadruplicate horizontally								
	1	2	3	4	1	2	3	4
A	POS1				POS2			
B	CRP				IFN-gamma			
C	IL-1 alpha				IL-1 beta			
D	IL-2				IL-4			
E	IL-6				IL-8			
F	IL-10				IL-12			
G	IL-17				MIP-1 alpha (CCL3)			
H	MMP-9				MMP-13			
I	Osteoprotegerin				Osteopontin			
J	Osteoactivin				RANK			
K	TGF beta 1				TNF-alpha			



X. Standard Concentrations

After reconstitution, the lyophilized cytokine standard mix contains the following concentrations for each antigen included.

Serial standard concentration (pg/ml)

(pg/ml)	Cntrl	Std7	Std6	Std5	Std4	Std3	Std2	Std1
CRP	0	55	165	494	1,481	4,444	13,333	40,000
IFNγ	0	14	41	123	370	1,111	3,333	10,000
IL-1α	0	3	8	25	74	222	667	2,000
IL-1β	0	3	8	25	74	222	667	2,000
IL-2	0	5	16	49	148	444	1,333	4,000
IL-4	0	3	8	25	74	222	667	2,000
IL-6	0	3	8	25	74	222	667	2,000
IL-8	0	1	2	5	15	44	133	400
IL-10	0	3	8	25	74	222	667	2,000
IL-12	0	3	8	25	74	222	667	2,000
IL-17	0	14	41	123	370	1,111	3,333	10,000
MIP-1α	0	14	41	123	370	1,111	3,333	10,000
MMP-9	0	14	41	123	370	1,111	3,333	10,000
MMP-13	0	27	82	247	741	2,222	6,667	20,000
OPG	0	27	82	247	741	2,222	6,667	20,000
OPN	0	137	412	1,235	3,704	11,111	33,333	100,000
Osteoactivin	0	14	41	123	370	1,111	3,333	10,000
RANK	0	137	412	1,235	3,704	11,111	33,333	100,000
TGFβ1	0	137	412	1,235	3,704	11,111	33,333	100,000
TNFα	0	3	8	25	74	222	667	2,000

XI. Spiking & Recovery

The antibody pairs used in the kit have been tested to recognize their specific antigen. The spiking and recovery rates of each cytokine in 2x diluted serum (SR), plasma EDTA (PLE), plasma citrate (PLC), plasma heparin (PLH), and culture media (CM) are listed in the following tables.

The spiking recovery rate for culture media and serum

(pg/ml)	Spiking	CM	CM + Ag	CM%	Serum	Serum + Ag	Serum%
CRP	20,000	0	14,411	72%	7,444	20,945	68%
IFNγ	5,000	391	4,425	81%	27	3,607	72%
IL-1α	1,000	21	746	72%	10	654	64%
IL-1β	1,000	0	631	63%	0	513	51%
IL-2	2,000	432	2,536	105%	49	2,152	105%
IL-4	1,000	14	1,244	123%	10	1,361	135%
IL-6	1,000	1,726	2,436	71%	27	1,023	100%
IL-8	1,000	159	1,156	100%	6	956	95%
IL-10	1,000	9	726	72%	1	590	59%
IL-12	1,000	2	1,198	120%	2	857	85%
IL-17	5,000	0	3,461	69%	2	3,255	65%
MIP-1α	5,000	110	7,134	140%	0	3,934	79%
MMP-9	5,000	15	2,587	51%	3,368	4,067	14%
MMP-13	10,000	129	9,919	98%	6	6,128	61%
OPG	10,000	34,602	39,009	44%	23	12,799	128%
OPN	50,000	958	47,043	92%	203	28,861	57%
Osteoactivin	5,000	89	4,993	98%	2,493	4,538	41%
RANK	25,000	68	19,119	76%	21	17,503	70%
TGFβ1	50,000	313	54,434	108%	0	60,182	120%
TNFα	1,000	42	765	72%	16	1,301	128%

XII. Quantibody® Q-Analyzer

The Q-Analyzer is an array specific, Excel-based program. It is much more than a simple calculation macro; it performs sophisticated data analysis (see below for description).

The Q-Analyzer Tool specific for this array is catalog number: **QAH-PDD-1-SW**.

Key features:

- Simplicity: Easy to operate and requires no professional training. With a simple copy and paste process, the cytokine concentration is determined.
- Outlier Marking & Removing: The software can automatically mark and remove the outlier spots for more accurate data analysis
- Normalization: The program allows for intra- and inter-slide normalization for large numbers of samples.
- Two Positive Controls: The program utilizes the two positive controls in each array for normalization.
- Two Analytical Algorithms: Users can choose either linear regression or log-log algorithms to meet their analytical needs.
- Two Data Outputs: standard curves and digital concentration.
- User Intervention: The program allows for user manual handling of outliers and other analytical data.
- Lower and Upper Limits Determination: The program automatically marks out the values below or above the detection range.
- Standard Deviation: The program outputs the standard deviations of the quadruplicate spots for data accuracy.
- Analytical Tips: Q-Analyzer analysis tips are included in the program.

XIII. Troubleshooting Guide

Problem	Cause	Recommendation
Weak Signal	Inadequate detection	Increase laser power and PMT parameters
	Inadequate reagent volumes or improper dilution	Check pipettes and ensure correct preparation
	Short incubation time	Increase incubation time or change sample incubation step to overnight
	Too low protein concentration in sample	Lessen dilution or do not dilute sample. Concentrate sample if necessary.
	Improper storage of kit	Store kit as suggested temperature. Don't freeze/thaw the slide.
Uneven signal	Bubble formed during incubation	Decrease amount of rocking during incubations. check for bubble formation and remove bubbles.
	Arrays are not completed covered by reagent	Completely cover arrays with solution for all required steps.
	Reagent evaporation	Cover the incubation chamber with adhesive film during incubation
Poor standard curve	Cross-contamination from neighboring wells	Avoid overflowing wash buffer and other solutions into neighboring wells.
	Comet tail formation	Air dry the slide for at least 1 hour before usage
	Inadequate standard reconstitution or Improper dilution	Reconstitute the lyophilized standard well at the room temperature before making serial dilutions. Check pipettes and ensure proper serial dilutions.
	Inadequate detection	Increase laser power so the highest standard concentration for each cytokine receives the highest possible reading yet remains unsaturated.
	Use freeze-thawed cytokine standards	Always use new cytokine standard vial for new set of experiment. Discard any leftover.
High background	Overexposure	Lower the PMT or sigmal gain.
	Dark spots	Completely remove wash buffer in each wash step.
	Insufficient wash	Increase wash time and use more wash buffer
	Dust	Work in clean environment
	Slide is allowed to dry out	Don't dry out slides during experiment.

XIV. Publications Citing This Product

1. Kinney JS, Morelli T, Oh M, Braun TM, Ramseier CA, Sugai JV, Giannobile WV. Crevicular fluid biomarkers and periodontal disease progression. J Clin Periodontol 2014; 41: 113-120. doi: 10.1111/jcpe.12194
   Species: Human
   Sample Type: Gingival Crevicular Fluid

2. Filho-Nogueira G., Pesun I., Ploegman C., Wijegunasinghe M., McCulloch C. ongitudinal Comparison of Cytokines in Peri-Implant Fluid and Gingival Crevicular Fluid in Healthy Mouths. Journal of Periodontology (2014)
   Species: Human
   Sample Type: Gingival Crevicular Fluid

More citations for this product may be available.
Contact techsupport@raybiotech.com.

XV. Experiment Record Form

Date:_____

File Name:_____

Laser Power:_____

PMT:_____

Well No.	Sample Name	Dilution factor
1	CNTRL	
2	Std7	
3	Std6	
4	Std5	
5	Std4	
6	Std3	
7	Std2	
8	Std1	
9		
10		
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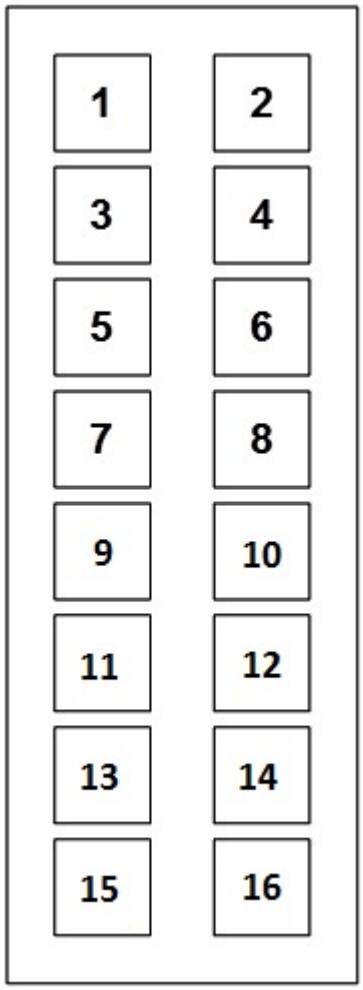

XVI. How to Choose a Quantibody® Array?

Species-based selection:

Human (QAH-)	Mouse (QAM-)	Rat (QAR-)	Bovine (QAB-)	Canine (QAC-)
Equine (QAE-)	Feline (QAF-)	Primates (QAN-)	Porcine (QAP-)	Rabbit (QAL-)

Function-based selection:

Adhesion Molecule Arrays	Angiogenesis Arrays	Bone Metabolism Arrays	Chemokine Arrays
Custom Arrays	Cytokine Arrays	Growth Factor Arrays	IGF Signaling Arrays
IL-1 Family Arrays	Immune Response Arrays	Inflammation Arrays	Interleukin Arrays
Isotyping Arrays	MMP Arrays	Obesity Arrays	Ophthalmic Arrays
Periodontal Disease Arrays	Receptor Arrays	Th1/Th2/Th17 Arrays	

Cytokine Number-based selection:
Arrays are available in the Quantibody® platform to detect 660 human, 200 mouse, or 67 rat proteins. GLP-Compliant testing services are also available.

To learn more about the Quantibody® Antibody Array, visit
www.RayBiotech.com/Quantibody-Multiplex-Elisa-Array.html

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